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DISEASE IN WILDLIFE OR EXOTIC SPECIES

# Fatal Disseminated *Toxoplasma gondii* Infection in a Captive Harbour Porpoise (*Phocoena phocoena*)

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## Summary

A 7-year-old female harbour porpoise (*Phocoena phocoena*), born and held in captivity, suffered from reduced consciousness, imprecise and circling swimming movements and long phases of immobility over a period of 3 weeks. The animal died during treatment in a Danish open sea facility. Pathological examination revealed multifocal pyogranulomatous to necrotizing meningoencephalomyelitis, ganglioneuritis, plexus chorioiditis, myocarditis, hepatitis and adrenalitis with few intralesional protozoal tachyzoites and bradyzoites within cysts. Immunohistochemistry was positive for *Toxoplasma gondii* antigen within the lesions. Using polymerase chain reaction (PCR), the presence of *T. gondii*-specific genome fragments was confirmed. A multilocus PCR-restriction fragment length polymorphism analysis using nine unlinked marker regions (nSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) resulted in the identification of *T. gondii* type II (variant Apico Type I), which is the *T. gondii* genotype dominating in Germany. This is the first description of disseminated fatal toxoplasmosis in a captive harbour porpoise that lived in an open sea basin. Surface water contaminated with toxoplasma oocysts is regarded as the most likely source of infection.



*Keywords:* encephalitis; harbour porpoise; polymerase chain reaction; *Toxoplasma gondii*

*Toxoplasma gondii* is a ubiquitous apicomplexan parasitic protozoon capable of infecting all warm-blooded animals including livestock, man and wildlife (Innes, 2010). Felids, including domestic cats, represent definitive hosts of *T. gondii* with a sexual parasitic phase in the feline intestine and an asexual phase in the intermediate hosts. *T. gondii* is transmitted horizontally via feline faeces and vertically from the infected animal to the fetus (Frenkel *et al.*, 1970; Dubey and Frenkel, 1972; Hill *et al.*, 2005). In mammals, infection with *T. gondii* can be either asymptomatic causing seroconversion or can lead to fatal systemic disease. Immunosuppression contributes to the development of systemic disease in *T. gondii* infections. In addition to terrestrial animals, infection with *T. gondii* causing morphological changes has been described in numerous marine mammals (Hill *et al.*, 2005; Dubey, 2008) including the striped dolphin (*Stenella coeruleoalba*; Di Guardo *et al.*, 2010), Beluga whale (*Delphinapterus leucas*; Mikaelian *et al.*, 2000), sea otter

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(*Enhydra lutris nereis*; Cole *et al.*, 2000), spinner dolphin (*Stenella longirostris*; Migaki *et al.*, 1990) and Risso's dolphin (*Grampus griseus*; Resendes *et al.*, 2002). Captive marine mammals including dolphins and sea lions (*Zalophus californianus*) have been reported to be infected with *T. gondii* (Migaki *et al.*, 1977; Dubey *et al.*, 2009). Serological evidence of *T. gondii* infection has been described in a single harbour porpoise (*Phocoena phocoena*; Cabezon *et al.*, 2004). In the present case, a fatal systemic infection with genotype II of *T. gondii*, a genotype prevailing in Germany, is described in a captive harbour porpoise.

The female porpoise was born on August 10th, 2007 at Fjord & Bælt, Kerteminde, Denmark, and died there spontaneously in April 2013. She was held with three other harbour porpoises in a netted sea pen in the harbour of Kerteminde. The other porpoises were her parents, caught in 1997 and a female, which was rescued as a juvenile in 2004. These three animals did not show any signs of disease during the period described in this article. The porpoise presented 3 weeks before death with reduced appetite and motivation to interact with the trainers. Complete blood count and serum biochemistry revealed low white blood cells (WBCs; $1.75 \times 10^9$/l; normal $3.5–7.0 \times 10^9$/l) and a high erythrocyte sedimentation rate (60 mm/h; normal <10 mm/h) suggestive of an acute infection. Serum activity of alanine aminotransferase (ALT) and γ-glutamyltransferase (GGT) was elevated (ALT: maximum = 1,000 U/l, normal <50 U/l; GGT: maximum = 153 U/l, normal <40 U/l) indicating liver damage. One week later the animal showed disorientation, bumping against the wall and tilting to its left side. Respiratory rate (10–15 breaths/min; normal <5 breaths/min) and WBC count (19th April: $10.1 \times 10^9$/l; 20th April: $7.3 \times 10^9$/l) were increased. The porpoise was treated continuously for suspected gastrointestinal blood loss with ranitidine and sucralfate. At the onset of clinical signs, treatment with enrofloxacin, amoxicillin and clavulanic acid was initiated, then stopped for 2 days and reinitiated 2 days before death due to signs of pneumonia. Due to the suspicion of lungworm infestation, the animal was treated with fenbendazole, clemastine and prednisolone (3.75 mg/day in 2009, 2011 and 2012).

A complete necropsy examination was performed as described by Lehnert *et al.* (2013). Formalin-fixed tissue samples were embedded in paraffin wax and sections (3–5 μm) were stained with haematoxylin and eosin (HE). For immunohistochemistry (IHC), a polyclonal rabbit antibody against *T. gondii* (Quartett, Berlin, Germany), a polyclonal rabbit antibody against tachyzoites of the NC-1 isolate of *Neospora caninum* (Schares *et al.*, 1997) and a polyclonal rabbit morbillivirus-specific antibody (panmorbilli, #25, kindly provided by C. Örvell, Central Microbiological Laboratory, Stockholm, Sweden) were applied using the avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Burlingame, California, USA) method with 3, 3′ diaminobenzidine tetrahydrochloride (DAB) as chromogen.

For the detection of Apicomplexan-specific genome fragments fresh, snap-frozen samples of heart, liver, lung and central nervous system (CNS) were examined using polymerase chain reaction (PCR). PCRs to characterize the presence of two *T. gondii* types were performed on DNA extracted from CNS tissue. A *N. caninum*-specific PCR was applied on DNA extracted from fresh frozen heart, liver and CNS samples. An overview of the primers used is given in Table 1. Genotyping of *T. gondii* DNA was carried out as described previously using genetic markers nSAG2, BTUB, GRA6, SAG3, c22-8, c29-2, L358, PK1 and Apico (Su *et al.*, 2006; Herrmann *et al.*, 2010). Three reference strains including RH, Me49 and NED were included in each PCR-restriction fragment length polymorphism (RFLP) run (Bretagne *et al.*, 1993; Yamage *et al.*, 1996; Magnino *et al.*, 1998; Reischl *et al.*, 2003).

At necropsy examination, the animal was in good nutritional condition with a body weight of 48 kg. Approximately 80% of both lung lobes showed dark red consolidation of the parenchyma. In the right lung lobe there were several dark grey nodules (up to 1 cm diameter). There was mild splenomegaly.

**Table 1**
**Overview of PCR assays used in this study**

| *Target organism* | *Target gene* | *Length of target fragment (base pairs)* | *Reference* |
|---|---|---|---|
| Selected *Coccidia* | *SSU-rRNA* gene | 294 | Magnino *et al.*, 1998 |
| *Neospora caninum* | *Nc-5* gene | 328 | Yamage *et al.*, 1996 |
| *Toxoplasma gondii* | *B1* gene | 115 | Bretagne *et al.*, 1993 |
| | 529 base pair DNA repeat element | 162 | Reischl *et al.*, 2003 |

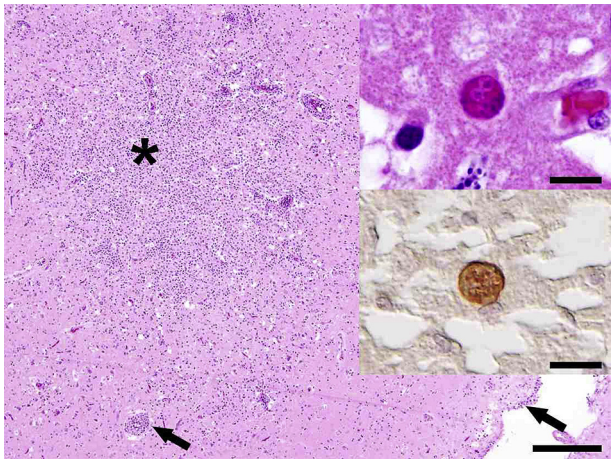

Fig. 1. Cerebrum of the harbour porpoise showing severe, focally extensive necrosis of grey and white matter with abundant gitter cells, neutrophils and macrophages (*). Multifocal perivascular cuffing (left arrow) and mononuclear infiltration of the meninges (right arrow) are present. HE. Bar, 200 µm. Upper inset: protozoal structures arranged in tissue cysts in the cerebrum. HE. Bar, 10 µm. Lower inset: immunohistochemical labelling of a protozoal tissue cyst in the cerebrum. Bar, 10 µm.

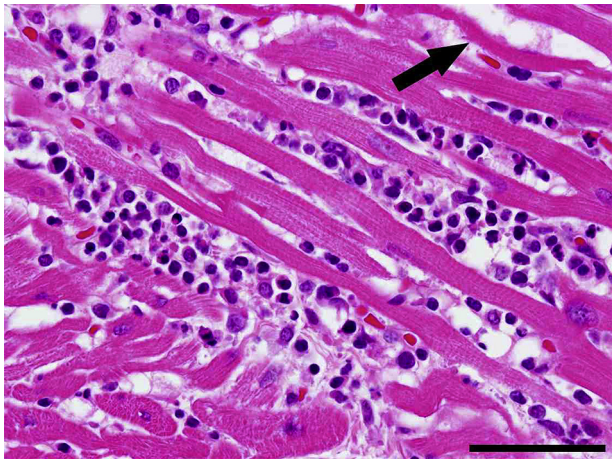

Fig. 2. Myocardium of the harbour porpoise showing multifocal moderate infiltration of neutrophils, macrophages and lymphocytes. Myofibres show loss of cross-striation and homogeneous, eosinophilic cytoplasm (myofibre degeneration and necrosis, arrow). HE. Bar, 100 µm.

Microscopically there was moderate, multifocal, subacute, pyogranulomatous, partly necrotizing meningoencephalomyelitis (involving the cerebrum, brainstem, cerebellum and spinal cord; Fig. 1) and plexus chorioiditis with gitter cells, astrogliosis, neuronal degeneration and necrosis. Intralesional tachyzoites and protozoal tissue cysts containing bradyzoites were detected in the myocardium and the CNS and were further identified by IHC (Fig. 1). The white matter showed few dilated myelin sheaths and spinal nerves were affected by mild, multifocal, lymphoplasmacytic neuritis and perineuritis. Necrotizing ganglioneuritis was present adjacent to the rete mirabile.

Multifocally, the septal and ventricular myocardium displayed moderate, multifocal, pyogranulomatous and necrotizing inflammation (Fig. 2) with myocardial degeneration and few intralesional protozoal organisms.

Mild to moderate, lymphoplasmacytic, interstitial pneumonia was present with mild alveolar histiocytosis. In the medial dorsal right lung there was chronic granulomatous pneumonia associated with intralesional nematode remnants and associated granulation tissue formation.

Pyogranulomatous to necrotizing inflammation was also present in the aortic wall, the liver (Fig. 3) and the muscular layer of the urinary bladder. Both adrenal glands showed foci of cortical necrosis. Portal and periportal areas of the liver also showed tissue necrosis, with portal fibrosis that was partly bridging. In the right ear there was mild focal, primarily lymphocytic ganglioneuritis of the spiral ganglion with solitary neuronal necrosis. Few perivascular and interstitial lymphocytes and macrophages were found in the dorsolateral musculature, diaphragm, oesophagus, left kidney, stomach, retina and large intestine. In the intestinal wall, inflammation was associated with ganglioneuritis and chromatolysis of intramural ganglion cells. No changes were present in the coeliac ganglion, thyroid and pituitary gland, small intestine, uterus, tonsils, skin of the dorsal fin, pulmonary lymph node, caudolateral spinal muscle and tongue. IHC for *N. caninum* antigen revealed slight somatic

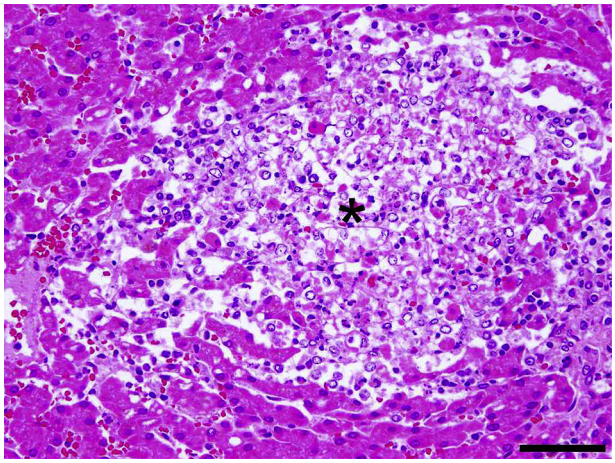

Fig. 3. Liver of the harbour porpoise showing focal pyogranulomatous to necrotizing hepatitis (*). HE. Bar, 50 µm.

labelling of intralesional tachyzoite-like structures. Morbillivirus antigen was not detected.

For Apicomplexa-specific genome fragments PCR was positive for the CNS and negative for heart, liver and lung. Both the B1 gene fragment and the 529 base pair fragment of the *T. gondii* genome were present in tissues of the CNS. *N. caninum*-specific genome fragments were not detected in any of the tested samples. Results of the PCR-RFLP analysis revealed *T. gondii* type II (variant Apico Type I).

This captive porpoise died due to systemic toxoplasmosis with pyogranulomatous to necrotizing inflammation, predominantly within the CNS, myocardium, liver and adrenal glands. Clinical and pathological changes caused by *T. gondii* share the spectrum of lesions described in marine mammals with systemic toxoplasmosis. *T. gondii*-induced protozoal meningoencephalitis is, beside *Sarcocystis neurona*, the second most detected protozoal parasite in brain lesions (Thomas *et al.*, 2007). In Californian sea otters (*Enhydra lutris*) *T. gondii* is able to act as a primary pathogen (Miller *et al.*, 2004). In other sea mammal species it has been suggested that *T. gondii* acts as a secondary pathogen after immunosuppression due, for example, to epidemics of morbillivirus or high levels of polychlorbiphenyls (Mikaelian *et al.*, 2000; Van Bressem *et al.*, 2009). In the present case, pathological results, including morbillivirus-specific IHC, did not suggest an underlying disease leading to immunosuppression. The severity of pulmonary parasite infestation was low and the lesions were chronic and did not suggest a clinically significant compromise of the immune system. It is possible that prolonged treatment with steroids may have contributed to systemic dissemination of *T. gondii*, although the treatment ended approximately 1 year before the death of the animal. All other animals in the same open sea basin were clinically normal and none died due to toxoplasmosis; although, nothing is known about seroconversion of the remaining animals. However, whether animals exposed to *T. gondii* will subsequently develop disease depends on the virulence of the parasite, the immune status and susceptibility of the host, the number of protozoal oocysts within the food or water and also the type of *T. gondii* (Di Guardo and Mazzariol, 2013). Furthermore, in marine mammals, environmental factors such as wind, tides, precipitation and water temperature contribute to developing disease (Miller *et al.*, 2004).

*T. gondii* oocysts remain viable for up to 18 months (Frenkel *et al.*, 1975), so the infection could have taken place up to 2 years before the onset of clinical signs. In the present case, it remains unknown whether the animal was infected *in utero*, early during life or shortly before clinical signs and death (Hill and Dubey, 2002).

Oysters living in water containing *T. gondii* may serve as a source of infection for marine mammals (Lindsay *et al.*, 2001). However, oocysts shed by domestic cats are regarded as important sources of infection for intermediate hosts and the detection of a *T. gondii* clonal type prevailing in German cats in the porpoise confirms the hypothesis that water was contaminated by oocysts that were most likely shed by domestic cats (Cole *et al.*, 2000; Schares *et al.*, 2008; Herrmann *et al.*, 2010).

In bottlenose dolphins and terrestrial animals, there is a classical life cycle for *T. gondii*, in which intermediate hosts ingest *T. gondii* oocysts, which are usually shed by faeces of infected cats (Dubey, 1998). A different pathogenesis is suggested for those animals living in the open sea, which may not overlap with the classical life cycle and is therefore referred to as the 'open sea *T. gondii* life cycle' (Di Guardo and Mazzariol, 2013).

PCR-RFLP analysis revealed *T. gondii* Type II (variant Apico Type I), which is the predominant *T. gondii* genotype found in faecal samples of German cats (Schares *et al.*, 2008; Herrmann *et al.*, 2010). In addition to the archetypal genotypes I, II and III of *T. gondii*, new genotypes called X and A have been described in sea otters (Howe and Sibley, 1995; Grigg *et al.*, 2001; Miller *et al.*, 2004, 2008; Sundar *et al.*, 2008). Possible explanations for the negative PCR results within the myocardium, liver and lung of the present case are firstly, an uneven distribution of *T. gondii* and secondly, low numbers of parasitic structures, such that nucleic acids in the tissues did not reach the level of detection of the assay.

In conclusion, the present case has shown that harbour porpoises are susceptible to systemic toxoplasmosis. For animals held in captivity, precautions should be implemented to minimize the risk of contamination of the water basin with infectious pathogens, in particular *Toxoplasma* spp. oocysts.

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## Conflict of Interest Statement

The authors declare no conflict of interest.

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